Variables determining the response of invertebrate species to toxicants, A case study on the River Meuse.
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Citation for published version (APA):

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Chapter VI

VARIABLES DETERMINING THE IMPACT OF DIAZINON ON AQUATIC INSECTS:
TAXON, DEVELOPMENTAL STAGE AND EXPOSURE TIME

Abstract
Several variables determine the impact of a pesticide on aquatic invertebrates. In this study, aquatic insects were subjected to the frequently occurring insecticide diazinon, analysing the variables taxon, developmental stage and exposure time. Effects of diazinon on the caddisfly *Hydropsyche angustipennis* and the midge *Chironomus riparius* were determined in the laboratory during different exposure times (48 and 96 h), using mortality, activity and growth as endpoints. Last instars of both species displayed a clear behavioural response at concentrations much lower than those affecting survival. Doubling the exposure time from 2 to 4 days decreased survival of midges and caddisflies by a factor 1.4 - 8.4. The 96 h LC50's were: 1.3 μg/L (caddisfly first instar), 29 μg/L (caddisfly fifth instar), 23 μg/L (midge first instar) and 167 μg/L (midge fourth instar). Within the spectrum of tested insects (9 species of which 48h LC50 are reported in literature), *H. angustipennis* is the second most sensitive, and *C. riparius* the most tolerant species. However, the ranking of species strongly depends on the developmental stage; differences between species are often smaller than differences between instars of one species. The large difference in sensitivities between young and old larvae imply that the impact of a pesticide strongly depends on the season of occurrence. Runoff from pesticide applications on crops is more likely to occur in spring and summer, and may have a relatively greater impact on insect communities since young larvae prevail in these seasons. In addition recovery of typical riverine insects like *H. angustipennis* from incidental exposure will be slow, considering their relatively long life cycle.
CHAPTER VI

Introduction

Although many sources of water pollution have been reduced in the past years, the input of pesticides into the environment remains critically high (RIWA, 1997; Lennox et al., 1998). A part of the pesticides applied in agriculture runs off into surface waters (Larson et al., 1995), becoming a potential risk for aquatic life. In addition to agricultural input, pesticides are released into rivers as a result of urban use (Burkhard and Jenson, 1993; Larson et al., 1995; Stamer and Wieczorek, 1996) and due to spills (Van Urk et al., 1993). Adverse effects of pesticides have been observed after chronic exposure to low pesticide concentrations (Schulz and Liess, 1995). In an international symposium reviewing the rehabilitation of the River Rhine, it was suggested that especially incidental spills are an important barrier for further improvement of the water quality (IKSR, 1996). Also in many other large rivers, "background" pesticide levels alternate regularly with concentration peaks (Van Meerendonk et al., 1994; Lennox et al., 1998). In the present study, the impact of short-term exposure to relatively high levels of pesticides on macroinvertebrates will be determined, in order to gain insight into how effects of these hazardous compounds are expressed.

There are several variables that determine the impact of a pesticide on invertebrate species. Sensitivity to toxicants is well known to differ between species, but much less well described is the variation due to developmental stage (Hutchinson et al., 1998). In addition, the response of an individual is largely determined by the exposure time to a toxicant (Anderson, 1989). Consequently, the susceptibility of a population may be largely determined by the timing and duration of pesticidal application (Van Wijngaarden, 1993).

The aim of this study is to evaluate the impact of an insecticide on different species that are closely related to the "target species". Aquatic insects were subjected to the frequently occurring (Burkhard and Jenson, 1993; Stamer and Wieczorek, 1996) insecticide diazinon, taking the variables taxon, developmental stage and exposure time into account. Effects of diazinon on two insects that commonly occur in rivers, the caddisfly *Hydropsyche angustipennis* and the midge *Chironomus riparius*, were determined in the laboratory during different exposure times, using mortality, activity and growth as endpoints. The observations were compared to literature data to develop the means to evaluate the potential risk of highly variable insecticide exposures in surface waters.
Materials and Methods

Chironomus riparius, first instars
Newly hatched midge larvae were obtained from a laboratory culture (Stuijfzand et al., 1998), and were distributed randomly in glass containers (180 mL), containing 110 mL of Dutch Standard Water (DSW). (DSW is a synthetic analogue of common Dutch surface waters (Maas et al., 1993).) After addition of the larvae (25 per container), food was added ad libitum, by supplying 0.5 mL of a food suspension (5 g Trouvit and 0.25 g Tetraphyll in 100 mL DSW) to each container. The containers were placed in an incubator, in which the temperature was kept at 20° C, and the light : dark regime was 16 : 8 h. The water was continuously aerated, and the containers were covered with plastic foil in order to prevent evaporation. The effects of diazinon on the midge larvae were determined after 48 h and 96 h, in separate experiments.

In the experiment which lasted 48 h, the following concentrations were tested in triplicate: 0, 1, 3, 10, 30, 50, 70, 100 μg diazinon/L DSW. During an exposure time of 96 h, the following concentrations were tested in triplicate: 0, 10, 15, 30, 50, 70 μg diazinon/L DSW. Half an hour after addition of diazinon and at the end of the experiments, water samples (10 mL) from each container were taken for analysis of actual diazinon concentrations. After 48 h or after 96 h, survival and activity and growth of the larvae were determined. Activity was considered to be "normal" when larvae were swimming, foraging or ventilating between rest periods, and swimming after a mechanical stimulus (touching them with a pipette). These larvae were easily distinguished from larvae displaying "abnormal" behaviour. The latter showed cramps, or were tightly curled, or showed almost no activity even after a mechanical stimulus. Growth was measured as well; at the start of each experiment, the lengths of 10 first instar larvae were measured using a binocular microscope. The average length of a newly hatched larvae was approximately 1 mm. At the termination of each study the lengths of the surviving larvae were measured. Growth was calculated by subtracting the average initial length from the final length of the individual larvae. Average growth of controls was always between 0.4 and 0.7 mm for the 48 h experiment, and between 1.0 and 1.1 mm for the 96 h experiment. Mortality of controls was 7.4 ±1.4%.

Chironomus riparius, fourth instars
Six egg ropes, obtained from a laboratory culture of C. riparius (Stuijfzand et al., 1998), were added to an aquarium containing DSW and clean sediment. The hatched larvae were raised and after two weeks, fourth instar larvae (of identical age) were selected from this mini-culture. The fourth instars were distributed randomly in glass aquaria (1.5 L), so that each aquarium contained 30 larvae. Before addition of the larvae, each aquarium was supplied with 150 mL glass beads (⌀: 2 mm) and 1 L of DSW. The larvae were fed ad libitum, by adding 1.5 mL of a Trouvit and Tetraphyll suspension (see above). The aquaria
were kept under identical conditions as mentioned above. The effects of diazinon on the midge larvae were determined after 48 h and 96 h, in separate experiments.

In the experiment in which the exposure time was 48 h, the following concentrations were tested: 0, 30, 100, 150, 250, 350, 450 µg diazinon/L DSW. The concentrations tested during 96 h of exposure were: 0, 10, 50, 100, 150, 200, 300 µg diazinon/L DSW. In both experiments, each treatment consisted of at least two replicates. Half an hour after addition of diazinon and at the end of the experiments, samples (10 mL) from each aquarium were taken for analysis of the actual diazinon concentrations in the water. After 48 h or 96 h, survival and activity of the larvae were determined, as described above. Mortality of control larvae was 1.9 ± 1.1%.

**Hydropsyche angustipennis, first instars**

First instar caddisfly larvae were obtained from a laboratory culture (Greve et al., 1998). Twelve day old larvae were distributed randomly in glass containers (180 mL), containing 110 mL of DSW. Each container held 20 larvae. After addition of the larvae, 0.5 mL of a food suspension (2.5 g dried and pulverized nettle (Urtica) in 100 mL DSW) was supplied. The containers were placed in an incubator, in which the temperature was kept at 20°C, and the light:dark regime was 16:8 h. The water was aerated, and the containers were covered with plastic foil in order to prevent diazinon on the caddisfly larvae were determined after 48 h and 96 h, in separate experiments.

In the experiment which lasted 48 h, the following concentrations were tested in triplicate: 0, 0.1, 0.3, 1, 3, 10 µg diazinon/L DSW. During an exposure time of 96 h, the following concentrations were tested (at least in duplicate): 0, 0.1, 0.3, 1, 2, 3, 4, 6, 8, 10 µg diazinon/L DSW. Half an hour after addition of diazinon and at the end of the experiments, water samples (10 mL) from each container were taken for analysis of the actual diazinon concentrations in the water. After 48 h or after 96 h, survival and activity of the larvae were determined. Activity was considered to be "normal" when larvae were foraging, swimming or ventilating between rest periods, and behaving "aggressively" after a mechanical stimulus (touching them with a pipette). These larvae were easily distinguished from larvae displaying "abnormal" behaviour. The latter showed cramps, or were tightly curled, or showed almost no activity even after a mechanical stimulation. Mortality of controls was 13 ± 2%.

**Hydropsyche angustipennis, fifth instars**

Fifth instar larvae were collected in the River Erft (near Cologne, Germany). Immediately after arrival in the laboratory, the larvae were distributed randomly in glass aquaria (1.5 L). Before adding the larvae, each aquarium was supplied with 150 mL glass beads (Ø: 2 mm) and 1 L of DSW. Each aquarium contained 10 larvae. The larvae were fed ad libitum, by adding 5 mL of an Urtica suspension (see above) to each aquarium. The aquaria were
kept under conditions as mentioned above. The effects of diazinon on the fifth instar caddisflies were determined after 48 h and 96 h within one experiment. The following concentrations were tested: 0, 10, 30, 90, 270 μg diazinon/L DSW. There were four replicates of each treatment. Half an hour after addition of diazinon and after 48 and 96 h, samples (10 mL) from each aquarium were taken for analysis of the actual diazinon concentrations in the water. After 48 h, survival and activity of the larvae were determined. After determining survival and activity, dead larvae were removed, and the larvae were fed 5 mL of food suspension again. Survival and activity of the larvae were determined again 96 h after the start of the experiment. Mortality of controls was 5 ± 5%.

Stock solution diazinon
A stock solution containing diazinon (O, O-diethyl O-[2-isopropyl-6-methyl-4-pyrimidinyl]phosphorothioate; 99.7% purity, Luxan, Elst, The Netherlands) was made using a generator column technique, following Bleeker et al. (1998). The actual concentration of the stock solution was 60 mg diazinon/L DSW.

Chemical analysis
Water samples were extracted with distilled hexane thrice, after addition of an internal standard (50 μl of 1.14 mg chlorpyriphos/L hexane). After extraction, the sample was concentrated by reducing the volume of hexane with nitrogen gas. Diazinon concentrations were measured by GC/MS (HP 5890), using a HP 5970 series Masse Selective Detector. The sample was injected cold on column (J&W Scientific, Folsom, CA, USA; type DB-5, 30 m length, 0.32 mm internal diameter, 0.25 μm film thickness) with helium gas as the carrier. The initial temperature was 80°C, the rate of temperature increase 20°C/min, and the final temperature was 274°C. The atomic mass unit selected was 304 for diazinon and 197 for chlorpyrifos. The actual diazinon concentration was determined by comparison with a standard containing a known diazinon concentration and internal standard concentration.

Results
Clear dose-response relationships between diazinon concentrations and survival of young larvae of both the midge and the caddisfly were observed (Fig. 6.1). The survival of the older larvae was more variable, but also decreased with increasing diazinon concentrations. From these dose-response relationships, LC50's and EC50's were calculated following Haanstra et al. (1985) (Table 6.1). It is clear that for both species, first instars die at much lower concentrations than last instars; the LC50's of young larvae were at least 7 times (midge) or even 23 to 84 times (caddisfly) lower than for older larvae.
It is also apparent that survival of *H. angustipennis* is affected at lower concentrations than that of *C. riparius*, and hence, first instar caddisflies were the most sensitive to diazinon.

![Graph showing effects of diazinon on survival of different instars](image)

**Fig. 6.1:** Effects of diazinon after 96 h exposure on survival of first and fourth instars of *C. riparius* and first and fifth instars of *H. angustipennis*.

After 48 h of exposure, the LC50 for fourth instars of the midge *C. riparius* could not be calculated (Table 6.1) since almost all larvae had survived the highest dose of diazinon (268 μg/L). However, a prolonged exposure time (to 96 h) led to mortality of more than 50% at this concentration (Table 6.1, Fig. 6.1). Similar observations were made with last instars of *H. angustipennis*; the LC50 decreased more than 8 times after prolonged exposure to diazinon. The LC50's of younger instars also decreased after 96 h compared to after 48 h, but this reduction was less pronounced.
Sublethal and lethal effects of diazinon on first instars of *C. riparius* were observed at similar concentrations (Fig. 6.2, Table 6.1; it should be noted that growth and activity are a percentage of the amount of surviving larvae, while survival is a percentage of the number of larvae at t=0). Older larvae, however, displayed a clear behavioural response at concentrations much lower than those affecting survival. While no mortality took place at the highest concentration (268 μg/L), the activity of the larvae was already affected at the lowest dose of diazinon (16 μg/L). These changes in activity of fourth instars were observed at similar or even lower diazinon levels than those at which first instars suffered from lethal effects (Fig. 6.2, Table 6.1).

![Figure 6.2: Effects of diazinon after 48 h exposure on survival and growth of first instars, and survival and activity of fourth instars of *C. riparius*.](image-url)
As for the first instars of *C. riparius*, sublethal and lethal effects on young *H. angustipennis* larvae due to diazinon were observed at similar concentrations (Fig. 6.3). Like *C. riparius*, last instars of the caddisfly showed a severe behavioural response at much lower concentrations than those at which mortality was observed. However, these concentrations at which activity of fifth instars was affected still seemed higher than those at which survival and activity of first instar larvae were affected (Fig. 6.3, Table 6.1).

![Fig. 6.3: Effects of diazinon after 48 h exposure on survival and activity of first and fifth instars of *H. angustipennis*.](image-url)
Table 6.1: LC50's and EC50's of diazinon for *H. angustipennis* and *C. riparius*. n.d.: not determined, *: could not be calculated.

<table>
<thead>
<tr>
<th>species</th>
<th>instar</th>
<th>exp. time</th>
<th>parameter</th>
<th>LC50 / EC50 (µg/L)</th>
<th>95% conf. limit</th>
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<tr>
<td><em>H. angustipennis</em></td>
<td>1</td>
<td>48</td>
<td>mortality</td>
<td>2.9</td>
<td>2.2 - 3.9</td>
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<tr>
<td><em>H. angustipennis</em></td>
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<td>96</td>
<td>mortality</td>
<td>1.3</td>
<td>1.2 - 1.5</td>
</tr>
<tr>
<td><em>H. angustipennis</em></td>
<td>1</td>
<td>48</td>
<td>activity</td>
<td>3.7</td>
<td>3.1 - 4.4</td>
</tr>
<tr>
<td><em>H. angustipennis</em></td>
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<td>96</td>
<td>activity</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>H. angustipennis</em></td>
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<td>48</td>
<td>mortality</td>
<td>242.8</td>
<td>123.1 - 478.9</td>
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<td>96</td>
<td>mortality</td>
<td>29.4</td>
<td>16.9 - 51.0</td>
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<td><em>H. angustipennis</em></td>
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<td>48</td>
<td>activity</td>
<td>14.5</td>
<td>10.3 - 20.5</td>
</tr>
<tr>
<td><em>H. angustipennis</em></td>
<td>5</td>
<td>96</td>
<td>activity</td>
<td>10.3</td>
<td>1.8 - 58.6</td>
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<td><em>C. riparius</em></td>
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<td>48</td>
<td>mortality</td>
<td>32.0</td>
<td>30.0 - 34.1</td>
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<tr>
<td><em>C. riparius</em></td>
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<td>96</td>
<td>mortality</td>
<td>22.8</td>
<td>19.7 - 26.3</td>
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<tr>
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<td>48</td>
<td>activity</td>
<td>22.6</td>
<td>4.8 - 105.8</td>
</tr>
<tr>
<td><em>C. riparius</em></td>
<td>1</td>
<td>96</td>
<td>activity</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>C. riparius</em></td>
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<td>48</td>
<td>growth</td>
<td>35.2</td>
<td>32.2 - 38.5</td>
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<td>31.7 - 103.7</td>
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<td>mortality</td>
<td>&gt; 268</td>
<td>*</td>
</tr>
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<td>96</td>
<td>mortality</td>
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<td>75.1 - 371.5</td>
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<td>48</td>
<td>activity</td>
<td>19.9</td>
<td>7.6 - 51.9</td>
</tr>
<tr>
<td><em>C. riparius</em></td>
<td>4</td>
<td>96</td>
<td>activity</td>
<td>17.9</td>
<td>15.9 - 20.2</td>
</tr>
</tbody>
</table>

Discussion

Pesticide sensitivity related to taxon, developmental stage and exposure time

The results clearly showed that the caddisfly *H. angustipennis* is much more sensitive to diazinon than the midge *C. riparius*. Within the spectrum of tested insects, *H. angustipennis* larvae are the second most sensitive, and *C. riparius* the most tolerant species (Fig. 6.4). However, among all insect species tested so far, there is no relationship between taxonomical groups (order, family) and sensitivity to diazinon. This is illustrated by the observation that the most tolerant and the most sensitive insect both belong to the genus *Chironomus*. On the phylum level, however, a general pattern can be discerned. When comparing insects, crustaceans and gastropods, it is apparent that arthropods (the first two groups) are the most sensitive to this insecticide (Fig. 6.4). The insensitivity of molluscs to diazinon was also observed in pilot experiments with zebra mussels, in which filtration rates were not affected after exposure up to 1000 µg/L (unpublished data). The available literature data on diazinon and the present study suggest that populations of arthropods like insects and crustaceans are likely to be the first macrofaunal groups damaged by diazinon discharges.
The results of the present study show that the ranking of species sensitivity is strongly dependent on the developmental stage; differences between species are often smaller than differences between instars of one species (Fig. 6.4). Of all reported 48 h LC50 values for insects, only in one case (Cloeon dipterum; Nishiushi and Asano, 1979) the age, weight or size of the test organism was mentioned. Given the strong dependency on the age or size in relation to the response of an organism as observed for both insect species in the present study, the (presented) ranking of sensitivity to diazinon using reported values is highly ambiguous.

Differences in sensitivity to organophosphorous insecticides between species can be quantitatively explained by differences in acetylcholinesterase inhibition (Legierse, 1998).
Enzyme activities of chironomids are affected by organophosphorous insecticides (Ankley and Collyard, 1995; Ibrahim et al., 1998), in contrast to those of the mollusc *D. polymorpha* (Dauberschmidt et al., 1997). Enzyme activities have also been observed to differ between developmental stages of insects (Ibrahim and Ottea, 1995), which may partly explain the differences in sensitivities to diazinon between first and last instars of one species. For fourth instars of *C. riparius*, acetylcholinesterase inhibition due to organophosphorous insecticides occurs at much lower concentrations than those at which mortality occurs (Ibrahim et al., 1998), and this inhibition strongly correlates with alterations in behaviour (Detra and Collins, 1991). Consistent with these observations, we found severe changes in activity of last instars of *C. riparius* and *H. angustipennis* exposed to low diazinon concentrations, while no mortality occurred.

Even a small increase in exposure time strongly increased toxicity to midges and caddisflies. Also for other pesticides, mortality was observed to increase with increasing exposure time (Länge et al., 1998; Legierse, 1998). In Fig. 6.5, an overview is given of macrofauna species that were exposed to diazinon at different exposure times (0-144 h). A steady decrease in LC50 occurs for all species with an increase in exposure time. Between 48 and 96 h, the LC50’s decrease on average by a factor 4.4 (± 5.0). The time dependency of effect concentrations is predicted by the pharmacological model of “target occupation” (Legierse, 1998). Another implication of this model is that lethal time increases with body size. The differences between responses of young and old larvae are consistent with Legierse’s [19] model. However, the observed effects are not only size-related; fifth instar caddisflies are larger than fourth instar midges yet they are more sensitive than the latter.

Aside from the present study, only one other study (Fernández-Casalderrey et al., 1994) reported an LC50 as well as an EC50 of diazinon for an invertebrate species. They observed that for *Daphnia magna* the 5 h EC50 for filtration rates was twice lower (0.47 μg/L) than the 24 h LC50 (0.9 μg/L). However, even within a shorter exposure period (5 h), EC50’s for filtration rates were twice lower (0.47 μg/L) than this LC50. This is in accordance with the present study, showing that last instars of *C. riparius* and *H. angustipennis* underwent severe behavioural effects at concentrations well below those at which mortality occurred. Sublethal effects may not immediately result in death in laboratory tests, but chances for survival will become extremely low when these effects occur in the field situation, where inactivity results in drift (Liess et al., 1993) and the chance on predation becomes higher.
Fig. 6.5: LC50's of different species related to exposure time to diazinon. The following taxa are presented: chironomids (*Chironomus riparius*: present study and *C. tentans*: Morgan, 1976), caddisflies (*Hydropsyche angustipennis*: present study and *H. sparna*: Morgan, 1976), a stonefly (*Pteronarcyis californica*: Cope, 1965), mayflies (*Cloeon dipterum*: Nishiushi and Asano, 1979 and *Paraleptophlebia pallipes*: Morgan, 1976), a daphnid (*Daphnia magna*: Mitchell, 1985), gammarids (*Gammarus lacustris*, *G. pseudolimnaeus* and *Hyalella azteca*: Morgan, 1976) and a gastropod (*Gilliia altifis*: Robertson and Mazzella, 1989) (References from AQUIRE).

Implications for macrofaunal populations in the field

The great difference in sensitivities between young and old larvae implies that the impact of a pesticide strongly depends on the season of occurrence. Diazinon is an insecticide that commonly occurs in waters throughout the year (Burkhard and Jenson, 1993; Kuivila and Foe, 1995; Larson et al., 1995). In some rivers, however, elevated pesticide levels are only detected in certain seasons. In the River Meuse (The Netherlands, Europe), all pesticide incidents (32 in total, concentrations ranging from 0.1 to 4.8 µg/L) during five consecutive years have been recorded in spring or summer (RIWA, 1993 - 1997). Since young larvae are especially abundant in these seasons (Hickin, 1967), these incidents are likely to have strong impacts. This implies that in late April, a diazinon peak in the River Meuse (0.6 µg/L; RIWA, 1997) had a greater impact on macrofauna populations than an incidental diazinon peak measured in February (1.1 µg/L; Kuivila and Foe, 1995) in the San Joaquin River (Arizona, USA), although the levels during the latter incident were
almost twice as high. In order to evaluate environmental risks of pesticide incidents, it is recommended to consider data from toxicity tests on young specimens since these organisms are often in the most sensitive phase in the life cycle of species.

In the River Meuse, 19 reports were made of incidental pesticide peaks during 1996 (RIWA, 1997). High concentrations of diazinon were detected twice (up to 0.6 µg/L; RIWA, 1997). During one of those incidental diazinon peaks, almost complete mortality (95-99%) of hydropsychid caddisflies (Stuijfzand et al., 1999) and chironomids (data not published) occurred upon exposure to Meuse water. The results obtained in the present study suggest that these diazinon peaks had a severe impact on macrofauna communities in the Meuse. Unlike C. riparius, H. angustipennis has disappeared from the Meuse since industrialisation, and has not yet returned to this river (Klink, 1985). The high sensitivity of first instar caddisflies to diazinon suggests that, even in the absence of other toxicants, the number of young H. angustipennis larvae, if present at all, would have been reduced in the River Meuse after the incidental discharges of diazinon.

Recovery of organisms after a pesticide peak depends on the taxon, the individual age or size, as well as on the length of the life cycle and other autecological characteristics (Van den Brink et al., 1996). Van Urk et al. (1993) reported that recovery of insects after the “Sandoz accident” (spills of several toxicants, among which organophosphorous insecticides) in the River Rhine took place after one or two generations. Univoltine species will take a longer period to recover than multivoltine species (e.g. Liess et al., 1993). Therefore, in addition to the negative effect of low diazinon concentrations, recovery of typical riverine insects like H. angustipennis will be slow, considering their relatively long life cycle. Since these species require stable conditions, they will not be able to maintain self-sustaining populations in rivers that are often affected by anthropogenic disturbances (Van Urk et al., 1993). In conclusion, even if the prevailing water quality of large rivers (like the Meuse) improves, frequently occurring incidents will prevent the return of typical riverine insects like caddisflies.

Acknowledgments The authors thank B. Scheper, M. Jonker and E. van Ammelrooy for practical assistance, P. Slot of the department of Environmental and Toxicological Chemistry (University of Amsterdam) for the analysis of diazinon, and W. Admiraal for critically reviewing the manuscript. This study was made possible through funds of the Institute for Inland Water Management and Waste Water Treatment and the Ministry of Housing, Spatial Planning and the Environment.
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